A platelet-activating factor antagonist reduces corneal allograft inflammation and neovascularization

Roberto A. Cohen, Bryan M. Gebhardt and Nicolas G. Bazan

Lions Eye Research Laboratories, LSU Eye Center, Louisiana State University Medical Center School of Medicine, New Orleans, LA 70112, USA

Abstract

We assessed the role of platelet-activating factor (PAF) in corneal allograft rejection and evaluated the effects of a PAF antagonist on corneal inflammation, cellular infiltration, vascularization, and edema. Rabbits with vascularized corneas served as recipients of allogeneic cornea grafts. Rabbits with normal corneas underwent autografts as controls. All of the allografts developed the progression of signs characteristic of rejection. Nevertheless, treatment with the PAF antagonist BN52021 significantly inhibited corneal allograft vascularization for up to 10 days after transplantation and reduced the number of eosinophils in the allografts at 28 days after transplantation. In contrast, saline-treated allografts exhibited florid vascularization and intense inflammatory infiltrates. Control autografts survived without developing significant inflammation or vascularization. The retardation of allograft eosinophilia and graft vascularization by the PAF antagonist was most likely the result of suppression of PAF-mediated reactions in the cornea. These results indicate that PAF may play a role in corneal inflammation and vascularization after corneal transplantation, and that PAF antagonists may be clinically useful in delaying some of the pathophysiologic consequences of corneal graft rejection. Curr. Eye Res. 13: 139−144, 1994.

Key words: platelet-activating factor; corneal allografts; inflammation; rabbits; corneal allograft rejection; corneal neovascularization; platelet activating factor antagonist

Introduction

Chemotherapeutic prevention and/or treatment of corneal inflammation following injuries, burns, infections, and surgery are critical to the preservation of vision and maintenance of normal corneal function. Corneal cells have been reported to produce mediators of inflammation, in particular platelet-activating factor (PAF) (1−3), which accumulates in the cornea after injury and is postulated to be a mediator of intraocular inflammation (3,4). Additionally, cells and infectious agents that infiltrate the cornea may also produce factors that elicit damaging inflammatory reactions (5−8). One of the consequences of corneal inflammation is neovascularization (9−13), which can result in permanent damage to the cornea (5,14) especially after repeated episodes (15,16).

Inflammation and vascularization after corneal transplantation may signal the onset of corneal allograft rejection resulting from the recognition of alloantigens by host lymphocytes and the destruction of graft cells (17−20). If this process cannot be modulated or reversed, the graft may fail. An agent that could reduce or reverse the inflammatory response in corneal grafts would be useful in the treatment of this clinically important phenomenon.

PAF has been shown to be involved in graft rejection (21,22), and the use of a PAF antagonist has been shown to mitigate the rejection response in at least one model (22). Although the role of PAF in the corneal model of injury and inflammation has been examined (1−5), little is known about the effect of PAF antagonists on corneal graft rejection.

Materials and methods

Animals

Twenty-four adult New Zealand white (NZW) and ten Dutch belted rabbits of both sexes were used as recipients and donors, respectively. NIH guidelines on the care and handling of animals in research (DHEW Publication, NIH 86-23) and the tenets of the Declaration of Helsinki were strictly adhered to throughout this study.

PAF antagonist

BN52021 (provided by Dr. Pierre Braquet, Institut Henri Beaufour, Paris) has been shown to act as a specific PAF antagonist in numerous models of PAF action (23,24). For this study, BN52021 was dissolved in sterile saline at a concentration of 20 mg per ml. *In vitro* and *in vivo* toxicity and pharmacokinetic studies in our laboratories (unpublished data) indicated that this concentration was optimal for use in these experiments.

Table 1. Clinical scoring system for grading corneas

Parameter	Grade			
	0	1	2	3
Cloudiness	Clear cornea	Hazy cornea, iris and iris vessels easily seen	Cloudy cornea, iris difficult to see, prominent features still apparent	Opaque cornea, iris not visible
Edema	Normal thickness cornea	Slightly thickened cornea	Significantly edematous cornea	Failed corneal graft
Corneal vascularization	No vessels in graft	Vessels entering graft in superior 120°	Vessels into graft from 180° to 240°	Vessels into graft from 360°

Corneal transplantation and PAF antagonist treatment

The study design necessitated 28 days of observation and treatment. Previous work (20,25) revealed that graft rejection would occur within this time frame, allowing us to collect data throughout the rejection process.

The upper halves of the corneas of 20 NZW rabbits (one eye each) were vascularized by placing five loops of 6-0 silk sutures in the superior 180° of the cornea at approximately one-half to two-thirds the depth of the corneal stroma, as described by Hill and Maske (20). Approximately two weeks after suture placement, all of the corneas were vascularized equally in the superior 180° of the cornea. Two days before the scheduled corneal transplantation, the sutures were removed and the corneas were allowed to heal. A standard corneal transplantation technique as reported previously from this laboratory (26) was employed. In brief, 7-mm, full-thickness corneal buttons were removed from the central cornea of graft recipients and replaced by 7.5 mm diameter corneal allografts from Dutch belted donor rabbits. The 10-0 nylon sutures were left in place throughout the 28-day observation period. Graft recipients were randomly assigned to the drug-treated or saline-treated group.

Four NZW rabbits (one eye each) that did not have placement of silk sutures underwent corneal autografting and were used as controls for comparison with the treated and untreated allografts.

At the time of surgery, and daily thereafter for 27 days, 10 of the 20 eyes receiving full thickness corneal allografts were given 6 mg of BN52021 in 0.3 ml as a subconjunctival injection using a 25-gauge needle. The other 10 allografted eyes received an equal volume of sterile saline subconjunctivally on the same schedule. The injection sites for the drug-treated and saline-treated groups were identical. Back flow was minimal and averaged less than 5% of the injected volume. Injections were performed under local anesthesia. The four autografted eyes received no injections.

All eyes were examined by slit lamp biomicroscopy and photographed every day and immediately before the animals were sacrificed 28 days after transplantation. The corneas were graded separately for cloudiness, edema, and neovascularization by two unbiased, independent observers. A clinical grading system with values ranging from zero (none) to 3 (most severe) was used to score the clinical condition of the grafts (Table 1). The data were analyzed using SAS Version 6.0 (SAS Institute, Inc., Cary, North Carolina). Individual observer variability was less than 3% of the means of the scores and variability between the two observers was less than 5% of the means of the scores.

Assessment of allograft rejection

Corneal graft reactions were scored using the grading system of Chen et al. (25). In this system, grade 4 graft reactions were established as endstage, irreversible rejection reactions. Such grafts were edematous, vascularized for 360°, and completely opaque.

Corneal histology and immunohistochemistry

Twenty-eight days after surgery, all animals including the autografted controls were sacrificed and the tissues were examined histologically and immunohistochemically. The corneas were removed, preserved in neutral buffered formalin for 24 hours, incubated an additional 24 hours in sterile 20% sucrose solution, frozen in Optimum Cutting Temperature (OCT) medium (Miles Laboratories, Shawnee, Kansas), and sectioned at 7 μm in a cryostat. Representative tissue sections were processed using the Luna technique (27) for the staining eosinophils and additional sections were stained for thymus derived (T) lymphocytes and Class II-expressing cells.

The immunohistochemical staining procedures were used as previously reported (28,29). Briefly, separate tissue sections were incubated in monoclonal antibodies specific for rabbit T lymphocytes (clone L11/135, secretes IgG_1 antibody, American Type Culture Collection, Rockville, Maryland) and Class II-expressing histocompatibility markers of the rabbit (Class II$^+$, clone 2C4, secretes IgG_2 antibody, American Type Culture Collection, Rockville, Maryland). The binding of the monoclonal antibodies in the tissue sections was revealed using the avidin biotin complex staining sequence (Vectastain, Vector Laboratories, Inc., Burlingame, California).

Tissue sections from six different levels of corneal allografts and autografts were examined for the presence of eosinophils, T lymphocytes, and Class II$^+$ cells. A masked and unbiased observer performed routine light microscopic evaluation of the six sections from each cornea and recorded the density of eosinophilic, lymphocytic, and Class II$^+$ cells in each section. Quantitative analysis of the cells that infiltrated the tissue sections was also performed using an image analysis system. The sensitivity of the image detector and background staining levels were established using sections from the autograft controls. Having established the zero parameters, we were then able to obtain density measurements across the entire length and width of the tissue sections. The T cells and class II$^+$ cells stained uniformly dark brown enabling us to obtain reproducible data. The eosinophils with azurophilic granules gave density readings that could be quantified in the tissue sections. Areas of the allograft sections that lacked T cells, class II$^+$ cells, or eosinophils did not contain stained artifacts that could have obscured the results of the density measurements.

Densimetric measurements of stained eosinophils, T cells, and Class II$^+$ cells in the tissue sections were performed using a Videoscope CCD 200E camera (Videoscope Int., Washington, DC) attached to a Nikon Optiphot microscope. Six sections consisting of both peripheral and central portions of each graft were examined. Eosinophils, T cells, and Class II$^+$ cells were identified in tissue sections, the images were digitized and the information stored in an Everex 486/25 computer using an Optimas Bioscan Program (Bioscan, Inc., Edmonds, Wash). Computer-assisted measurements of cell numbers as reflected by the density, the area stained, and the total area of the corneal sections permitted us to compare the cellular infiltrates in the treated and untreated groups. The densities of T cells, class II$^+$ cells, and eosinophils in three sets of six tissue sections from six different levels of each graft were densitometrically determined. The data obtained for T cells, class II$^+$, and eosinophils in the saline-treated and PAF antagonist-treated eyes were pooled into the two treatment groups and the mean percent of the tissue occupied by each cell type in each treatment group was determined. The data were analyzed using SAS Version 6.0 (SAS Institute, Inc., Cary, North Carolina).

Results

Clinical observations

None of the four control, autografted eyes showed significant cloudiness, edema, or vascularization, indicating that the transplantation surgery was not responsible for these conditions in the allografted eyes. All of the allografts underwent rejection over the 28-day period of the study.

Allograft rejection

Immune-mediated rejection of corneal allografts in vascularized graft beds occurred within 28 days, confirming previous reports (20,25). Grafts in saline-treated eyes and BN52021-treated eyes exhibited similar mean survival times (Table 2). Endothelial rejection lines were seen in five (two saline-treated and three BN52021-treated) grafts.

Table 2. Corneal allograft immune reactions in PAF antagonist-treated and saline-treated corneas

Treatment	Total no. of grafts	Time to first sign of graft reaction* (days)	Mean ($\pm$ SD) survival time (days)
Saline	10	15/2, 18/3, 20/1, 24/3, 25/1	20.1 $\pm$ 3.8
BN52021	10	14/1, 17/3, 21/2, 22/3, 24/1	19.7 $\pm$ 3.2

*First appearance of a corneal allograft immune reaction characterized by faint corneal haze and mild infiltrative vascularization at the original site of placement of the silk sutures. Numbers are days after surgery/number of grafts showing first signs of immune reaction on that day. SD, standard deviation.
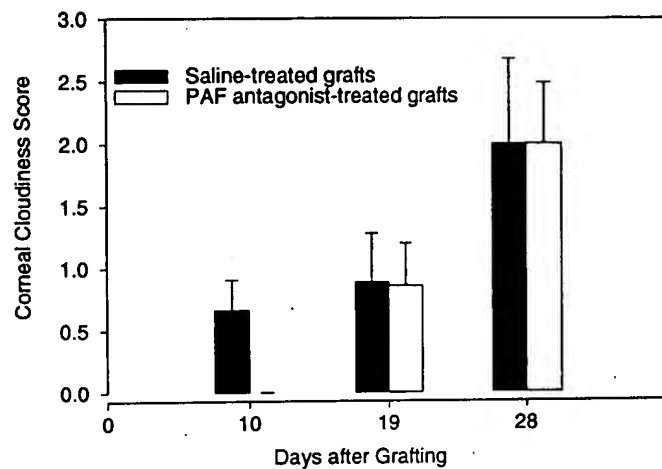

Figure 1. Corneal haze and cloudiness was scored from 0 to 3 by two independent observers using a scale in which 0 indicated a clear graft and 3 a completely opaque graft. The mean scores $\pm$ standard deviations of the PAF antagonist-treated and saline-treated eyes reveal a significant difference at 10 days (P < 0.005), but not at 19 or 28 days.

Corneal cloudiness

Corneal allografts treated with the PAF antagonist, BN52021, exhibited little or no corneal cloudiness in the first 10 days after grafting (Fig. 1), compared with noticeable cloudiness in the saline-treated corneal allografts as early as day 10. The difference between the cloudiness of antagonist-treated and saline-treated allografts was statistically significant (P < 0.005). By day 19 and thereafter, however, there was no difference between the saline-treated allografts and the BN52021-treated allografts in terms of corneal cloudiness (Fig. 1).

Corneal edema

BN52021-treated corneal allografts did not exhibit edema until day 14 after grafting, whereas the saline-treated allografts exhibited grade 1 edema as early as day 10. At day 10 there was
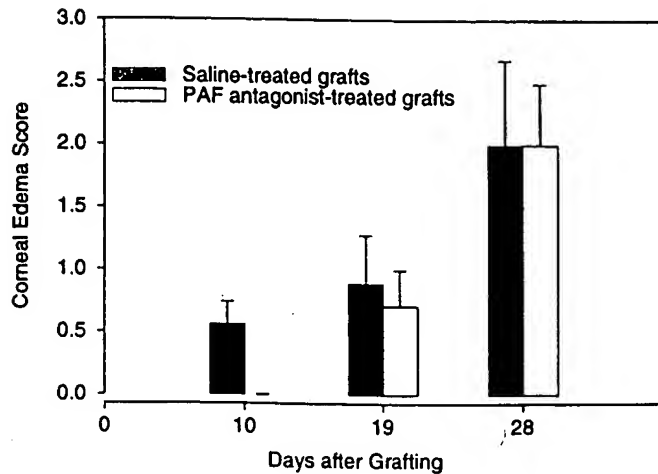
Figure 2. Corneal edema was measured from 0 to 3 by two independent observers using a scale in which 0 represented no edema and three represented a graft that was twice the normal thickness. The mean scores and standard deviations reveal a significant difference at 10 days (P<0.005), but not at 19 or 28 days.
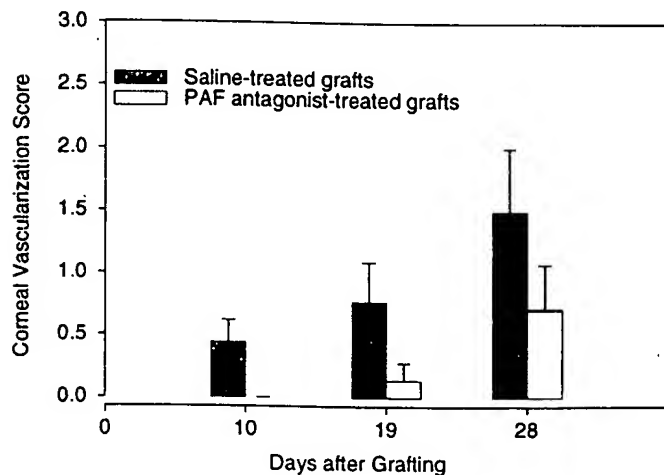

Figure 3. Corneal vascularization was scored from 0 to 3 by two independent observers using a scale in which 0 indicated no vascularization and 3 indicated a graft with vascularization in all quadrants. The mean scores and standard deviations reveal a significant difference at 10 days (P<0.05), but not at 19 or 28 days.

a statistically significant difference between the BN52021-treated corneas and the control corneas (P < 0.005). This difference was no longer apparent by day 19 and throughout the remainder of the observation period. On day 28 both the control and the antagonist-treated allografts exhibited similar degrees of corneal edema (Fig. 2).

Corneal neovascularization

The PAF-antagonist-treated corneas were significantly (P < 0.05) less vascularized on days 10 and 19, compared with the saline-treated corneas (Fig. 3). On day 28, vascularization
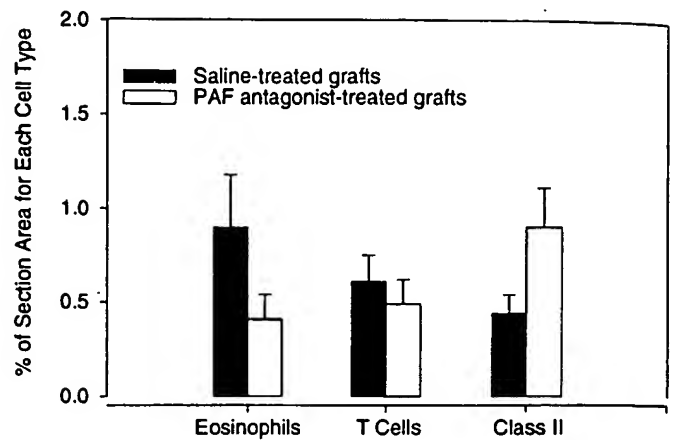

Figure 4. Corneal cell infiltrates were quantified by computerized image analysis of tissue sections obtained 28 days after grafting. The means and standard deviations for the area of the section imaged for each cell type in PAF antagonist-treated and saline-treated grafts are shown. There were significantly fewer eosinophils in the sections from the PAF antagonist-treated grafts compared with the sections from the controls (P<0.05), but no difference in the number of T cells. Increased numbers of Class II $^+$ cells in sections from antagonist-treated grafts were noted (P<0.05).

in the PAF antagonist-treated corneas was less than in the saline-treated group although the differences were no longer statistically significant.

Histology and immunohistology of corneal allografts

Image analysis quantification showed fewer eosinophils (0.91 ± 0.29 vs. 0.47 ± 0.18) and more Class II $^+$ cells (0.92 ± 0.31 vs. 0.48 ± 0.13) in the tissue sections from corneal allografts treated with the PAF antagonist, compared with the saline-treated allografts (Fig. 4). No differences were seen in the numbers of or percent of tissue area occupied by T lymphocytes (0.64 ± 0.18 vs 0.50 ± 0.17).

No correlation between the nature or intensity of the cellular infiltrate and the day of graft rejection was observed.

Discussion

PAF is a potent mediator of inflammatory reactions and platelet aggregation and has a variety of effects in mediating cell-to-cell interactions (23,24). It has been demonstrated that corneal epithelial cells produce PAF (3,4). In addition, when the cornea becomes inflamed and infiltrated by polymorphonuclear leukocytes and eosinophils, PAF levels greatly increase (3,4), and it is known that these two cell types are major sources of PAF (5,30). In the cornea, PAF potentiates the activities of acute inflammatory cells, causing leukocyte activation, release of leukocyte enzymes, and tissue disruption (3–5). If this action is extensive enough, the cornea becomes irreversibly edematous and damaged.

It is also known that the presence of inflammatory cells, especially polymorphonuclear leukocytes and eosinophils, stimulates corneal neovascularization (9–11,31,32). However, the

exact mechanisms by which leukocyte infiltrates induce neo-vascularization of an avascular tissue are not known.

We found that treatment with the PAF antagonist retarded the development of corneal cloudiness and edema, and reduced the development of vascularization of corneal allografts. The demonstration that corneal neovascularization can be decreased by treatment with a PAF antagonist suggests a role for PAF in the early phases of the immune graft reaction. The transient nature of the inhibitory effect of the PAF antagonist on the inflammatory response may be related to the dosage and route of administration. In order to be clinically useful, the antagonist may require sustained delivery in higher concentrations for a longer period of time.

The increased numbers of Class II⁺ cells in PAF antagonist-treated grafts requires further study. At the present time we do not know if these cells are new residents of the cornea graft or are graft cells that have been induced to express the Class II histocompatibility marker. The role of PAF in modulating Class II marker expression has not been studied.

·The most important observation of this study is that treatment of corneal allografts with a PAF antagonist resulted in reduced numbers of eosinophils in the allografts that underwent rejection. PAF is a powerful chemotactic agent for eosinophils (23). BN52021, a PAF antagonist, decreased the number of eosinophils in corneal grafts, suggesting that the effect is receptor-mediated. Although the role of eosinophils in corneal graft rejection has not been determined, the presence of these cells could modulate the immune response. A relationship between severe immune deficiency and eosinophilia has been noted in familial reticuloendotheliosis (23).

In this study, the PAF antagonist was administered via one daily subconjunctival injection. This treatment regimen was used in order to prolong the effect of the drug and to avoid manipulation of the graft such as occurs with intragraft injection. Studies are in progress to define the most effective routes of treatment and schedules of drug application to maximize the effect of PAF antagonists during corneal allograft rejection.

Acknowledgements

References

1. Bazan, H.E.P., Birkle, D.L., Beuerman, R. and Bazan, N.G. (1985) Inflammation-induced stimulation of the synthesis of prostaglandins and lipoxygenase-reaction products in rabbit cornea. *Curr. Eye Res.* **4**, 175–179.

2. Bazan, H.E.P. and Bazan, N.G. (1984) Composition of phospholipids and free fatty acids and incorporation of labeled arachidonic acid in rabbit cornea. Comparison of epithelium, stroma and endothelium. *Curr. Eye Res.* **3**, 1313–1319.

3. Bazan, H.E.P., Reddy, S.T.K., Woodland, J.M. and Bazan, N.G. (1987) The accumulation of platelet activating factor in the injured cornea may be interrelated with the synthesis of lipoxygenase products. *Biochem. Biophys. Res. Commun.* **149**, 915–920.

4. Bazan, H.E.P., Reddy, S.T.K. and Lin, N. (1991) Platelet-activating factor (PAF) accumulation correlates with injury in the cornea. *Exp. Eye Res.* **52**, 481–491.

5. Bazan, N.G. and Bazan, H.E.P. (1990) Ocular responses to inflammation and the triggering of wound healing: Lipid mediators, proto-oncogenes, gene expression, and neuromodulation. In 'New Trends in Lipid Mediators Research', Vol. 5, (Eds. Braquet, P. and Robinson, L.). Pp. 168–180. S. Karger, Basel, Switzerland.

6. Zimmerman, G.A., Whatley, R.E., McIntyre, T.M. and Prescott, S.M. (1987) Production of platelet-activating factor, a biologically active lipid, by vascular endothelial cells. *Am. Rev. Respir. Dis.* **136**, 204–207.

7. Elstad, M.R., Prescott, S.M., McIntyre, T.M. and Zimmerman, G.A. (1988) Synthesis and release of platelet-activating factor by stimulated human mononuclear phagocytes. *J. Immunol.* **140**, 1618–1624.

8. Vivier, E., Denizot, Y., Benveniste, J. and Thomas, Y. (1989) PAF-acether: Biosynthesis by bacteria and immunoregulatory functions. In 'Cell Activation and Signal Initiation: Receptor and Phospholipase Control of Inositol Phosphate, PAF, and Eicosanoid Production', (Eds. Dennis E.A., Hunter, T., and Berridge, M.). Pp. 337–344. Alan R. Liss, Inc., New York.

9. Fromer, C.H. and Klintworth, G.K. (1975) An evaluation of the role of leukocytes in the pathogenesis of experimentally induced corneal vascularization. I. Comparison of experimental models of corneal vascularization. *Am. J. Pathol.* **79**, 537–554.

10. Fromer, C.H. and Klintworth, G.K. (1975) An evaluation of the role of leukocytes in the pathogenesis of experimentally induced corneal vascularization. II. Studies on the effect of leukocytic elimination on corneal vascularization. *Am. J. Pathol.* **81**, 531–544.

11. Fromer, C.H. and Klintworth, G.K. (1976) An evaluation of the role of leukocytes in the pathogenesis of experimentally induced corneal vascularization. III. Studies related to the vasoproliferative capability of polymorphonuclear leukocytes and lymphocytes. *Am. J. Pathol.* **82**, 157–170.

12. McCracken, J.S., Burge, P.C. and Klintworth, G.K. (1979) Morphologic observations on experimental corneal vascularization in the rat. *Lab. Invest.* **41**, 519–530.

13. Schanzlin, D.J., Cyr, R.J. and Friedlaender, M.H. (1983) Histopathology of corneal neovascularization. *Arch. Ophthalmol.* **101**, 472–474.

14. Howes, E.L., Cruse, V.K. and Kwok, M.T. (1982) Mononuclear cells in the corneal response to endotoxin. *Invest. Ophthalmol. Vis. Sci.* **22**, 494–501.

15. Srinivasan, B.D. and Eakins, K.E. (1979) The reepithelialization of rabbit cornea following single and multiple denudation. *Exp. Eye Res.* **29**, 595–600.

16. Smolin, G. (1985) Inflammation of the external eye. *Drug Dev. Res.* **6**, 235–243.

17. Inomata, H., Smelser, G.K. and Polack, F.M. (1971) Corneal vascularization in experimental uveitis and graft rejection: An electron microscopic study. *Invest. Ophthalmol.* **10**, 840–850.

18. Abbott, R.L. and Forster, R.K. (1979) Determinants of graft clarity in penetrating keratoplasty. *Arch. Ophthalmol.* **97**, 1071–1075.

19. Alldredge, O.C. and Krachmer, J.H. (1981) Clinical types of corneal transplant rejection: Their manifestations, frequency, preoperative correlates and treatment. *Arch. Ophthalmol.* **99**, 599–604.

20. Hill, J.C. and Maske, R. (1988) An animal model for corneal graft rejection in high-risk keratoplasty. *Transplantation,* **46**, 26–30.

21. Khirabadi, B.S., Foegh, M.L., Goldstein, H.A. and Ramwell, P.W. (1987) The effect of prednisolone, thromboxane, and platelet-activating factor receptor antagonists on lymphocyte and platelet migration in experimental cardiac transplantation. *Transplantation,* **43**, 626–630.

22. Makowka, L., Chapman, F.A., Cramer, D.V., Qian, S., Sun, H. and Starzl, T.E. (1990) Platelet-activating factor and hyperacute rejection. The effect of a platelet-activating factor antagonist, SRI 63–441, on rejection of xenografts and allografts in sensitized hosts. *Transplantation,* **50**, 359–365.

23. Braquet, P. and Vargaftig, B.B. (1986) Pharmacology of platelet activating factor. *Transplant. Proc.* **18**, 10–19.

24. Braquet, P.L. (1987) The ginkgolides: Potent platelet-activating factor antagonists isolated from *Ginkgo biloba* L.: Chemistry, pharmacology and clinical applications. *Drugs of the Future,* **122**, 643–699.

25. Chen, Y.F., Gebhardt, B.M., Reidy, J.J. and Kaufman, H.E. (1990) Cyclosporine-containing collagen shields suppress corneal allograft rejection. *Am. J. Ophthalmol.* **109**, 132–137.

26. Newton, C., Gebhardt, B.M. and Kaufman, H.E. (1988) Topically applied cyclosporine in azone prolongs corneal allograft survival. *Invest. Ophthalmol. Vis. Sci.* **29**, 208–215.

27. Luna, L.G., ed. (1968) *Manual of Histologic Staining Methods of the Armed Forces Institute of Pathology. Third edition.* pp. 111–112. McGraw-Hill, New York.

28. Gebhardt, B.M. and Hill, J.M. (1988) T lymphocytes in the trigeminal ganglia of rabbits during corneal HSV infection. *Invest Ophthalmol Vis. Sci.* **29**, 1683–1691.

29. Gebhardt, B.M. and Hill, J.M. (1990) Cellular neuroimmunologic responses to ocular herpes simplex virus infection. *J. Neuroimmunol.* **28**, 227–236.

30. Braquet, P., Shen, T.Y., Touqui, L. and Vargaftig, B.B. (1987) Perspectives in platelet-activating factor research. *Pharmacol. Rev.* **39**, 97–145.

31. Ben Ezra, D. (1979) Neovasculogenesis. Triggering factors and possible mechanisms. *Surv. Ophthalmol.* **24**, 167–176.

32. Moore, J.W. III and Sholley, M.M. (1985) Comparison of the neovascular effects of stimulated macrophages and neutrophils in autologous rabbit corneas. *Am. J. Pathol.* **120**, 87–98.